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TITLE:   An Unconventional Approach To Reducing Retinal Degeneration After Traumatic Ocular Injury


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**14. ABSTRACT**
This project seeks to image abnormal retinal pericyte vasospasms after ocular trauma in mice, and their relationship to: retinal macrophage activity, and oral administration of L-Arginine. In the first year we overcame significant technical difficulties with in vivo fluorescent labelling of retinal pericytes, using both fiber-optic confocal and multiphoton microscopic imaging techniques, and we successfully established the imaging protocol for in vivo retinal pericyte imaging with simultaneous measurement of microspasms in retinal capillary beds.

**15. SUBJECT TERMS**
Mural cells, pericytes, vasospasms, traumatic ocular injury, L-Arginine, mice.

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1.　**INTRODUCTION:** The *objective* of this research is to determine if clinically available blood flow regulating drugs – currently not purposed to treat retinal damage – may serve to ameliorate retinal degeneration in mice who have experienced blast-related traumatic injuries. We will meet this objective through the use of novel in vivo imaging techniques along with a tested animal model developed specifically for blast-type ocular injury. Our *hypotheses* are based upon evidence that the vascular effects contribute to neurodegeneration after traumatic ocular injury. We will test whether neurodegeneration may be driven by microvascular dysfunction after an overpressure wave. We will also test the effects of oral administration of L-Arginine—a nitric oxide (NO) precursor—on ameliorating these vascular contributions. Blood flow control has traditionally been thought to be regulated by arteries, with the last point of active flow control taking place at the pre-capillary sphincters that connect arterioles to primary capillaries. Capillary beds, it follows, irrigate tissue passively: they are driven up and down in their flow uniformly, as a function of their arteriolar supply. The outcome of this model is that when an artery's flow is pathologically occluded—for example, by a stroke or by ocular injury—all of the flow in the downstream capillary beds is reduced uniformly—as only those neurons very near to microvessels have access to the dwindling oxygen supply—with severe neurodegeneration occurring in watershed areas farthest from vessels. If, instead, our novel model is correct, and individual microvessel mural cells (e.g. pericytes) vasospasm and contribute to neurodegeneration, we predict that neurodegenerating cells will be found predominantly nearby to (spatially associated with) capillary blood vessels, rather than in watershed areas. If this innovative model is correct, it will represent a transformative shift in the field's understanding of cardiovascular function in the eye, to include the capillary beds as the last point of blood flow control, rather than the arterioles.

2.　**KEYWORDS:** Mural cells, pericytes, vasospasms, traumatic ocular injury, L-Arginine, mice.

3.　**ACCOMPLISHMENTS:**

### 3.1 What were the major goals of the project in Year 1?
The main task to be accomplished in year 1 of this project was to select the ocular trauma model to test the project's hypotheses. This included the establishment of protocols for labelling cells and recording (imaging) techniques, as well as the measurement of baseline function in neurotypical mice.

　　Specific Aim 1:
　　　　Major Task 1: Select Ocular Trauma Model
　　　　　　Subtask 1: Conduct Fiber-optic imaging to determine blood-flow abnormality.
　　　　　　Subtask 2: Conduct Fiber-optic imaging to determine macrophage abnormality.

### 3.2 What was accomplished under these goals?

### 3.2.1 Major Activities

The major activities for the first year was to setup the new dual-band fiber-optic confocal microscope system, test it, and to establish the protocols for the first successful in vivo retinal microvessel and pericyte advanced microscopic imaging recordings. In achieving this, we detected the first retinal microvessel vasospasms in neurotypical subjects, at rest.

### 3.2.2 Specific Objectives Achieved

Our specific objectives were to establish the protocols to conduct these recordings in the retina, which we achieved successfully. We also successfully conducted control recordings in neurotypical mice, and recorded normal microvascular vasospasms for the first time.

### 3.2.3 Significant Developments, Results, and Conclusions

We purchased, receive, set up, tested and calibrated the new dual-band microscope. We then replicated our previously successful neural imaging methods of pericyte vasospasms, now in the retina instead of the brain, and found—to our surprise—that they failed (the remainder of year 1 was spent modifying these methods until this problem was rectified, as described fully below). We were not certain if the problem was hardware, software, fluorescent dye protocol, surgical methods, a more technical (analytical) problem concerning the new system (i.e. a programming issue), or—more concerning—that the retinal biology might simply be different from in the brain. So that our pericyte labelling system, which we developed in the brain simply might not function with retinal pericytes.

This last possibility, the biological one, was the potentially most vexing, so we immediately addressed this one, as soon as we realized that the experiments were not going to work without modification. The basis of the biological problem could have been that our in vivo pericyte dye (10kD fluorescent dextran) did not label retinal pericytes despite labeling brain pericytes, So we first verified that our animals' brain pericytes were indeed being stained properly. In doing so we discovered that brain pericytes were also not readily detectable using our new dual-band microscope. This failure to replicate in the brain left open a couple of possibilities, including the new hardware, methodological problems (i.e. perhaps the staff involved—who were all new since we just moved to a new institution from Phoenix to NYC—were not performing at the very high level necessary to achieve these recordings. And even if these problems were solved, this did not rule out the possibility that retinal pericytes were different than brain pericytes.

To narrow down the possibilities, we set up a new imaging experiment, now with two-photon microscopy, which used the postmortem retinas from the same mice used in the in vivo fiber-optic experiments. The idea was that, if the dyes were working correctly, but were simply too dim for the new dual-band fiberscope, then we would nevertheless see them with two-photon imaging, which is much higher quality. This worked well, and we not only replicated our previous results that the mouse brain pericytes and blood vessels were indeed being stained properly, but we found that the retinal pericytes were also being labelled by our novel pericyte staining method, just like brain pericytes (**Fig 1**). This success suggested that the problems we had had with retinal replications of pericyte vasospasms to date were either hardware or software problems, or perhaps issues that required optimization of methods, but that our fundamental methods were sound and that retinal pericytes were not fundamentally different than brain pericytes, in terms of the biology.

Now that we had established that the problems we were having were not biological in their basis, we set out to determine the whether the problem was due to the specific dyes we were using, or the hardware (or some other issue). Our previous experiments used a 2-meter long

fiber-optic objective, rather than the 6m fiber we were currently using. And the vast majority of our experiments used a single-band microscope rather than a dual-band microscope (which we had used only on loan to acquire preliminary data for this grant application). To address these possible sources of signal degradation, we tested the signal-to-noise ratios of fluorescein dextran at different dilutions (dose concentrations) as a function of both our single-band and dual-band scopes, and as a function of fiber length (**Fig 2**).
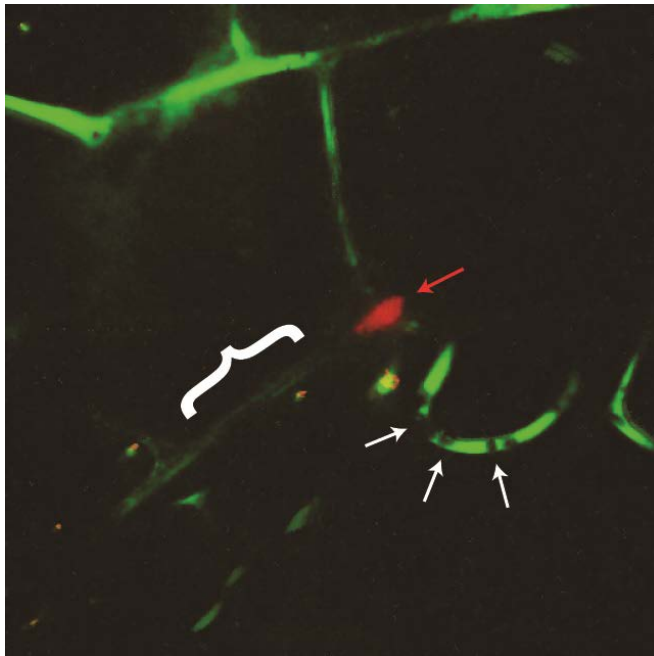


**Figure 1.** In vivo retinal labelling of pericytes (red fluorescent dextran—Red Arrow) in capillaries labelled with 2MD fluorescein dextran in the serum. The White Arrows point to three donut-shaped red blood cells within a capillary, indicating the high quality of imaging we were able to acquire from our in vivo staining methods. Note that the pericyte lies at the anastomosis of that same (White Arrows) vessel and another vessel descending from the microvessel at the top of the image. It appears that the pericyte lies at the same point that these vessels were blocked from feeding a third vessel that continues to the left of the pericyte (weakly stained White Bracketed vessel). As we previously showed in the brains of epileptic mice, this is a hallmark of pericyte vasospasm and suggests that before the animal was sacrificed, that the pericyte in question has acted to restrict flow down the bracketed vessel (a function not previously known for retinal pericytes).
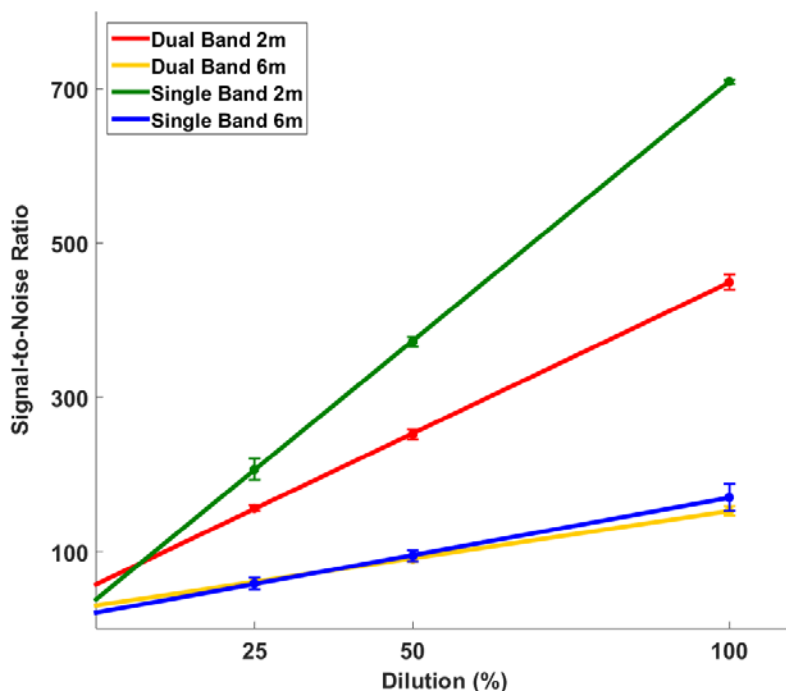


**Figure 2.** SNR as a function of fluorescein dextran dilution, for two microscopes (single-band Cellvizio vs dual-band Cellvizio), and two fiber-optic objectives (2m vs 6m). Notice that SNR was 3-7 times higher when using a 2m objective over a 6m objective. There is a SNR advantage to using a single-band microscope as well, but because our experiments require the measurement of two colors simultaneously we are restricted to using a dual-band for the remainder of our experiments.

These experiments suggested that, first, the dye protocol was good and the microscopes were functioning as promised because varying dilution resulted in linear changes in SNR. Second, we should use 2m probes instead of the 6m probes we had been using (we borrowed 2m probes from Mauna Kea Technologies to do these experiments and these experiments led to our decision to buy a set for for our experiments going forward). A parallel experiments of SNR experiments with red vs green dye (data not shown), moreover showed that green resulted in higher SNR in the dual-band, vs red dye, so we changed our staining protocol to use green dye for retinal pericytes (**Fig 3**), with red dye (angiosense) now used for blood serum staining (**Fig 4-5**). Now, using a 2m objective and green dye for the pericytes, we achieved strong retinal pericyte staining with simultaneous blood flow measurements.
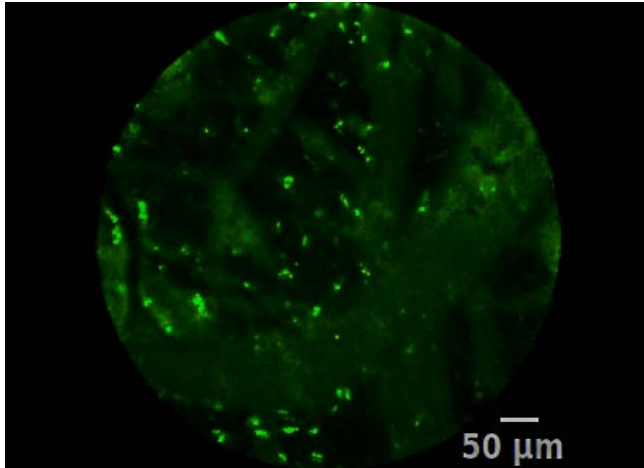


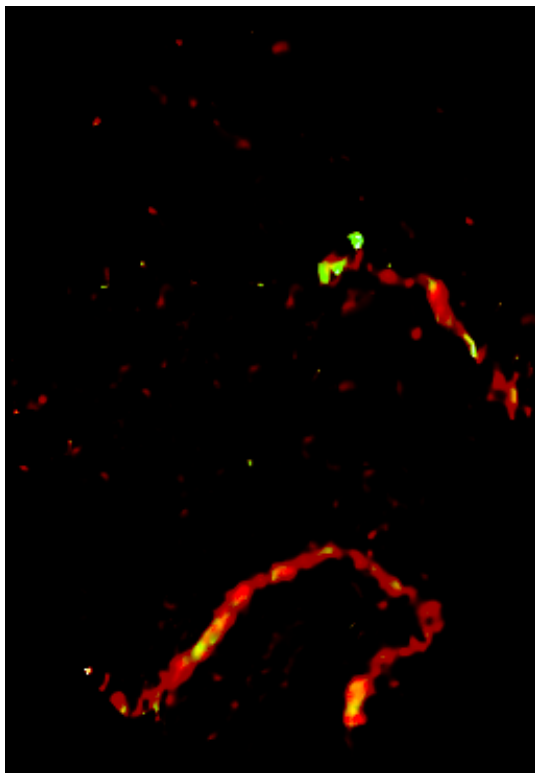**Figure 3.** The first successful in vivo retinal pericyte recordings.



**Figure 4.** Simultaneous retinal blood flow (red) and pericyte (green) images taken during in vivo fiber-optic recordings in a mouse.
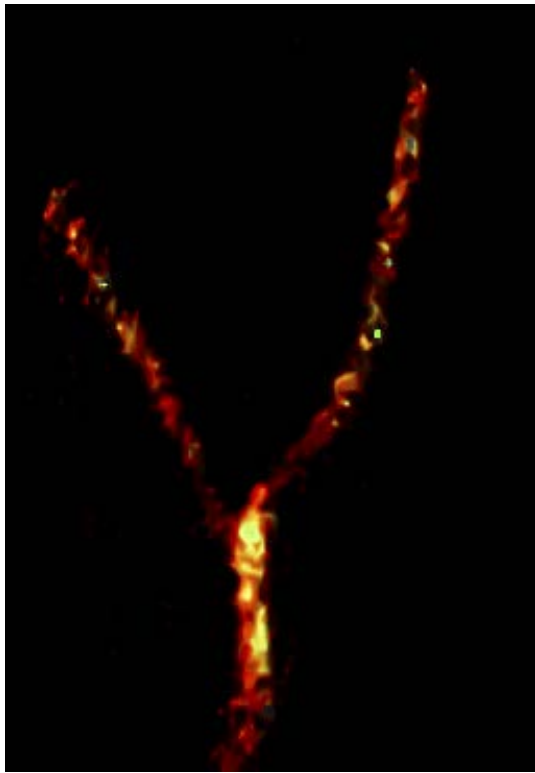
**Figure 5.** Simultaneous retinal blood flow (red) and pericyte (green) images taken during in vivo fiber-optic recordings in a different mouse than in Fig 4.

Now that we were successfully acquiring images of pericytes in the retina of healthy mice regularly, we began collecting the vasospasm data from our control (neurotypical mice; **Fig 6)**. The red arrows in both **Figure 6A** and **6B** point to a pericyte that is managing the flow of the vessel. When there is no flow in the vessel (**Figure 6A**) the pericyte, seen as a small red dot, constricts the flow of blood in the vessel. The fluorescence of that portion of the vessel decreases. As the (**Figure 6B**) flow into the vessel increases, the pericyte appears to increase in size (this is due to the vessel pressing the pericyte against the face of the fiber when irrigated). This behavior is quantified n subsequent figures.
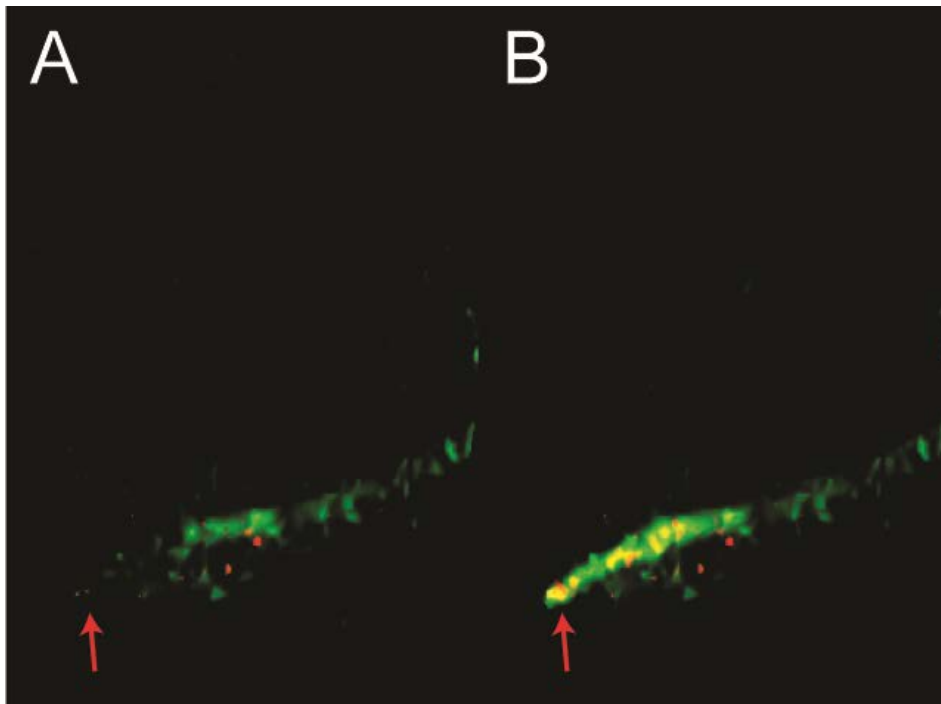


**Figure 6.** A pericyte driven vasospasm in a retinal capillary in a neurotypical mouse. (A) Red arrow indicates position of the pericyte but no flow (vasospasm), (B) Same red arrow shows the same pericyte no allowing free-irrigation of the microvessel.

Two Regions of Interest (ROIs) were selected from **Fig 6** and kept relatively constant throughout all recordings. The first ROI chosen was used to measure the intensities of pixels in a vessel that was vasospasming due to the constriction of pericytes attached to its outer walls. The second ROI measured the intensities of pixels in a vessel that was unchanging throughout all recordings. For the Fluorescence over Time graph (**Fig 7**), the initial raw signals were smoothened with a Savitsky-Golay Filter (order 5, 1501 frame size) and a Median Filter (order 3) to remove noise. Due to the fact that there was bleaching of the fluorescent dyes, both the red and green channels' luminosity slowly decreased over time (this is faster in pericytes because the dye is not renewed in those cells where as the bloodflow in vessels is constantly renewing the dye). In order to remove this drift, an order 51 polynomial curve was fit to the values of the unchanging vessel and then subtracted from both the data of the vessel with the vasospasms as well as the unchanging vessel. This removed the drift and made a baseline determined by the unchanging vessel. $\Delta F/F$ curves were determined by averaging an initial segment of each signal in order to get an F value for each of the vessels and then divide each point of the signal by that F and multiply by 100 to get a percentage fluorescence with respect to initial F value as seen in **Fig 7**. Vasospasms are clearly indicated with arrows that timestamp when they happened in the recording. Red arrows indicate complete vasospasms, while black arrows show partial vasospasmic behavior. In total, for the 8,750 seconds (around 2 hours and 25 minutes) of acquired data, 12 vasospasms were observed, 8 being complete and 4 being partial. On average, this vessel underwent a vasospasm every 729 seconds (around every 12 minutes).
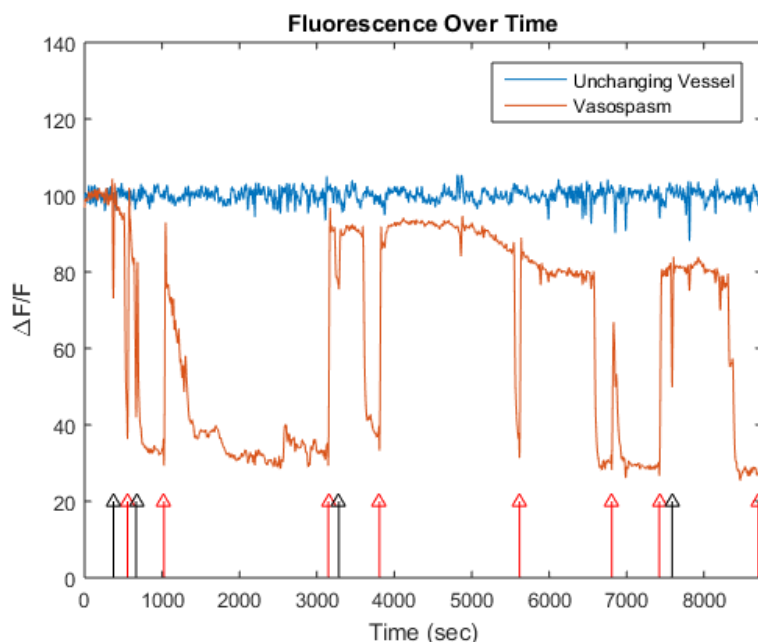


**Figure 7.** Two vessels simultaneously measured from Fig 6. $\Delta F/F$ curves measure the change in fluorescence in each ROI as a function of time. The blue vessel does not vary in fluorescence over time, which indicates that the changes we see in the orange channel are due to true vascular changes and not due to fiber motion artifacts (which would result in equivalent changes in both vessels). Red arrows indicate full vasospasm whereas black arrows indicate partial vasospasms.

Even though there were both partial and complete vasospasms in the data acquired, only the complete vasospasms were chosen for **Fig 8**. In each of these 8 vasospasms there is clear and stable fluorescence intensity in the ROI of the unchanging vessel, whereas the fluorescence of the ROI on the vessels that are spasming decreasse at an exponential rate. The signal in the vessels with vasospasms (shown in orange) are constant until a specific onset of the vasospasm and then their intensities decrease to about 40% of their initial values. The rate at which the vasospasms onset versus the rate at which they stop spasming is interesting to note. They decay relatively slowly compared to rate of the termination of the vasospasm, in which bloodflow returns to its initial value is extremely fast, almost instant. We assume this is due to blood pressure slowing the onset of vasospasm and likewise accelerating the release from vasospasm.
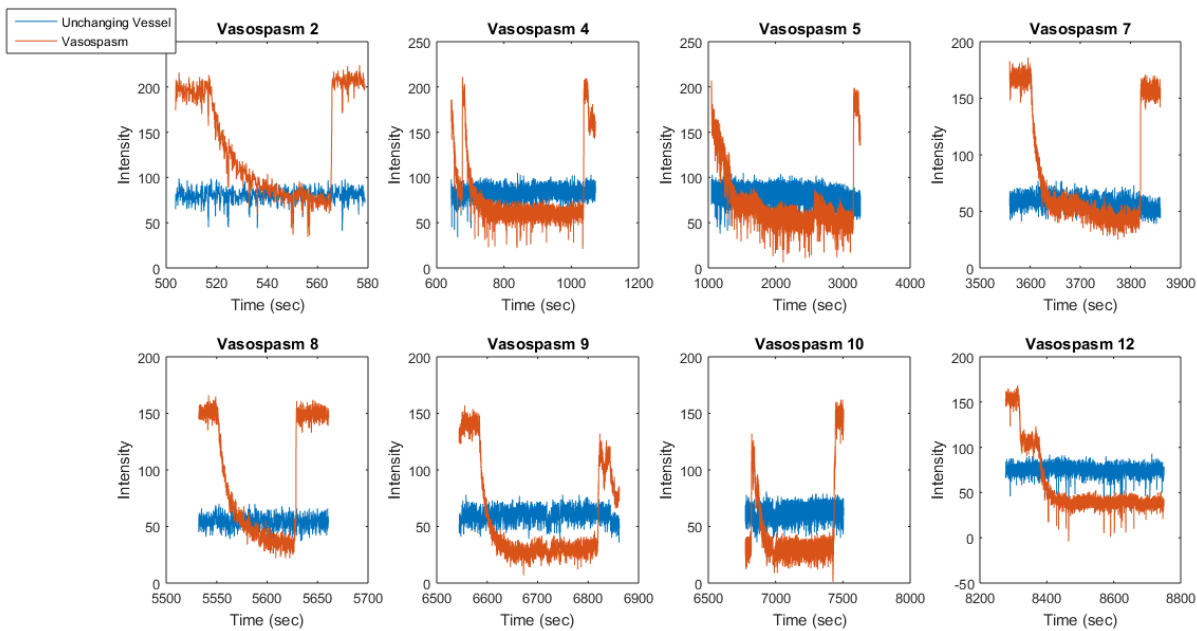
**Figure 8.** Eight full vasospasms from Fig 6 analyzed in detail. Notice the slow onset and fast termination of vasospasms, indicating that the pericytes closure interacts with extant blood pressure in these vessels.

We quantified the rate of onset (the tau of a fit exponential) versus the termination rate (**Fig 9**). The blood vessel opens at a higher rate than at which it closes. There is a significant difference ($p < 0.05$) between onset and termination taus.
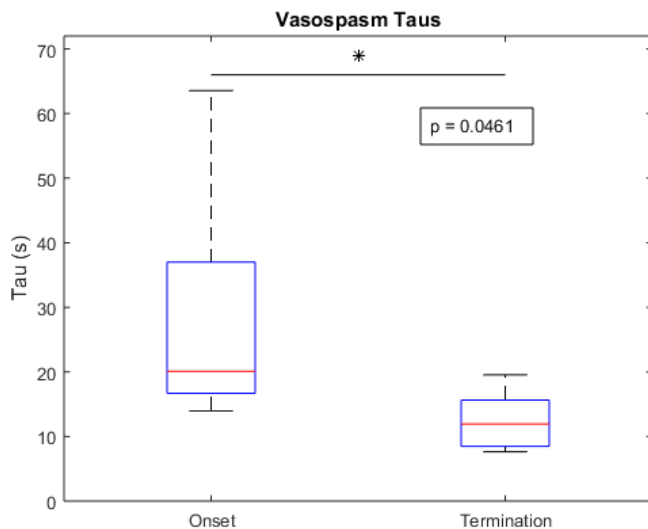


**Figure 9.** Onset versus termination rates of vasospasms from Fig 6.

### 3.2.4 Goals not met

We had hoped to complete the control experiments by now but due to a serious staffing problem in 2016 (see below), we were not able to do so. However, now that the staffing problems have been overcome, we expect to complete the control experiments by 2017, and also expect to complete the experiments with traumatic ocular injury mice before July 2017.

### 3.2.5 What opportunities for training and professional development has the project provided?

I had intended to train a graduate student (funded by a different source) with this project. Unfortunately, he had a serious mental breakdown (he was previously diagnosed with bipolar disease) and could not continue the experiments. This was a major setback for the project, though we have now trained new staff (who's time will be reported in FY 2).

### 3.2.6　How were the results disseminated to communities of interest?

*"Nothing to Report."*

### 3.2.7　What do you plan to do during the next reporting period to accomplish the goals?

In FY 2 we intend to complete the experiments and submit an abstract at the Society for Neuroscience conference 2017. We hope to publish in 2017 as well.

## 4　IMPACT: *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

*"Nothing to Report."*

## 5　CHANGES/PROBLEMS: *The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

### 5.2　Changes in approach and reasons for change

*See section 3 above.*

## 6　PRODUCTS:　*"Nothing to Report."*

## 7　PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

### What individuals have worked on the project?

*See section 3 above. Note that I cannot discuss details of the graduate student who left the program due to mental health concerns.*